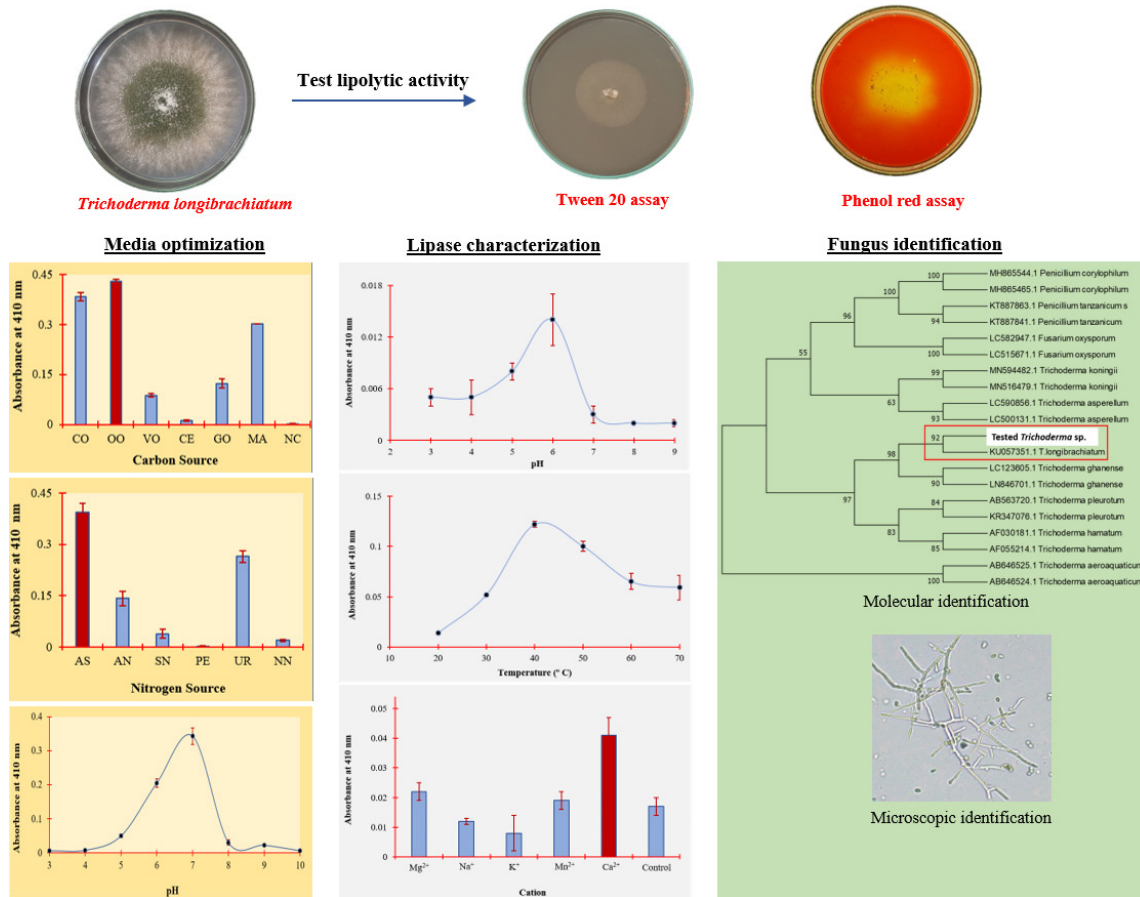**RESEARCH ARTICLE**

Identification of a lipolytic *Trichoderma* sp. and characterization of its extracellular lipase

N. K. Athukorala and P. Samaraweera

Highlights

- *Trichoderma longibrachiatum* shows lipolytic activity.
- Olive oil and ammonium sulfate increased *T. longibrachiatum* lipase secretion.
- The optimum pH for lipase secretion from *T. longibrachiatum* is 7.0.
- *T. longibrachiatum* lipase shows maximum enzyme activity at a pH 6.0 and 40 °C.
- Preincubation of *T. longibrachiatum* lipase with Ca^{2+} enhances its activity.

RESEARCH ARTICLE

Identification of a lipolytic *Trichoderma* sp. and characterization of its extracellular lipase

N. K. Athukorala* and P. Samaraweera

Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Sri Lanka.

Abstract: Lipases are abundantly used in a wide range of industries. Currently, microbial lipases are the most popular source of industrial lipases, of which fungal lipases stand out due to their low cost of production, catalytic activity, and extracellular nature. The demand for lipases has kept researchers exploring new lipolytic fungi. However, the success of such studies lies in the optimization of the growth medium to maximize lipase secretion and the characterization of the enzyme to achieve its highest activity. Therefore, this research was undertaken to identify a lipolytic fungus that was isolated from soil, optimize its lipase secretion, and characterize it. Based on the results of the current study, the lipolytic fungus was identified as *Trichoderma longibrachiatum*. Its lipase secretion was maximal when it was cultured in a medium containing olive oil and ammonium sulfate as carbon and nitrogen sources, respectively, at a pH of 7.0. Further, the *Trichoderma* lipase showed its highest activity at a pH of 6.0 and 40 °C. Moreover, preincubation of the lipase with Ca^{2+} increased its activity. The enzyme characterization revealed that the extracted *Trichoderma* lipase has industrially desirable properties. The research results showed that the lipolytic *Trichoderma longibrachiatum* could be focused on industrial applications.

Keywords: Enzyme characterization; fungal lipase; *ITS*; *Trichoderma longibrachiatum*.

INTRODUCTION

Lipases are acyl hydrolases that hydrolyze carboxylic ester bonds in triacylglycerols to release carboxylic acids and alcohols (Daiha *et al*., 2015). Lipases also catalyze other chemical reactions such as esterification, trans-esterification, aminolysis, and acidolysis (Mehta *et al*., 2017). Lipases show a folding pattern known as the α/β hydrolase fold that serves as a scaffold for the catalytic triad involved in lipase activity (Ollis *et al*., 1992). Lipases are an attractive and versatile group of enzymes in biotechnology due to their chemoselectivity, stereoselectivity, regioselectivity, availability, and independence from cofactors (Jaeger and Eggert, 2002). As forecasted by BCC (Business Communications Company) Research, the global market for lipases is projected to reach USD 797.7 million by 2025. Its wide application potential has encouraged researchers to search for novel lipases. Extensive research has been carried out to improve production and yield, understand the structural basis of enantioselectivity, and engineer the enzyme specificity of lipases (Gupta *et al*., 2015). Many years of studies on

lipases have not lessened the researchers' attention in this area. A study revealed that there is an increasing demand for these enzymes in kinetic resolution, the detergent industry, food and feed, and biodiesel production (Daiha *et al*., 2015).

Lipases are ubiquitous and thus found in microorganisms such as bacteria and fungi, plants, and animals. Lipases were traditionally extracted from the animal pancreas as a digestive aid for human consumption. Due to difficulties in practicalities and the extraction process, initial interest in microbial lipases was provoked (Hasan *et al*., 2006). At present, commercially available lipases are derived mostly from microorganisms (Schmid and Verger, 1998) and utilized in various industries such as food, paper, cosmetics, biodegradable polymers, pharmaceutical, textiles, detergents, and bioremediation (Hasan *et al*., 2006).

Fungal lipases distinguish themselves from other types of lipases and have gained interest from a variety of industries. This is due to their enantioselectivity, thermostability, stability under extreme pH, stability in organic solvents, ease of genetic manipulation, and low cost of production (Mehta *et al*., 2017). Lipases exhibiting such advantageous properties have already been identified in several fungi genera; some of them are *Trichoderma* (Ülker *et al*., 2011), *Aspergillus* (Liu *et al*., 2015), *Fusarium* (Gulati *et al*., 2005), *Penicillium* (Boratyński *et al*., 2018), *Rhizopus* (Helal *et al*., 2021), and *Geotrichum* (Burkert *et al*., 2005). When considering *Trichoderma* species, they are very efficient in making a wide range of extracellular enzymes (Harman, 2006). Studies have identified *Trichoderma* species producing lipases with industrially demanding properties. For instance, *Trichoderma harzianum* produces extracellular lipase with high stability at high temperatures and across a wide pH range (Ülker et al., 2011). Another study reporting on *Trichoderma lentiforme* lipase has revealed its high tolerance to both anionic and non-ionic surfactants (Wang *et al*., 2018). As a result, food waste contaminated soil was collected for this study in order to isolate the lipolytic *Trichoderma* sp. Food waste usually comprises a significant proportion of oil, thus there is a higher chance to isolate lipolytic fungi from such soil samples.

Several factors such as nitrogen source, carbon source,

*Corresponding Author's Email: nadeeshaa@sci.pdn.ac.lk

https://orcid.org/0000-0001-6486-2417

inorganic salts, temperature, pH, agitation rate, and dissolved oxygen concentration influence the production of lipases by microorganisms (Mehta *et al.*, 2017). Therefore, determining the sources of lipases and the proper conditions for enzyme production is crucial. Moreover, the need for understanding the factors that affect enzyme activity necessitates enzyme characterization experiments (Sharma *et al.*, 2001; Shahid *et al.*, 2011). With these understandings and demands, this study aimed to optimize the growth medium of lipolytic *Trichoderma longibrachiatum* to obtain a high lipase yield and optimize the conditions that result in high lipase activity. Further, the current research is intended to identify the properties that could make this lipase a suitable candidate for industrial applications.

MATERIALS AND METHODS

Initial culturing and characterization of the fungus

The uncharacterized lipolytic fungus, isolated from soil, was cultured on PDA (Potato Dextrose Agar) supplemented with 0.005% (w/v) ampicillin and spectinomycin antibiotics to obtain a pure culture. The point inoculated medium was incubated at 32 °C for three days. Initially, morphological characteristics were evaluated for 3-day old fungus cultures. Slide culture technique with LPCB (lactophenol in cotton blue) as the stain was used in microscopic identification. The three components of LPCB have different functions in the technique. Phenol kills the living microbes and cotton blue stains the chitin structures of the fungus, while lactic acid preserves the structure of the fungus (Leck, 1999). For molecular identification of the fungus, genomic DNA was isolated and amplified by PCR (Polymerase Chain Reaction) targeting the *ITS* region. The PCR product was then sequenced, and the resulting sequence was compared with known sequences available in databases.

DNA isolation

The isolation of the DNA of the lipolytic fungus was carried out using the cetyltrimethylammonium bromide (CTAB) method. The fungal mycelia (~100 mg) were scraped from a fresh culture plate into a 1.5 mL Eppendorf tube. The fungal mycelia were treated with 500 µL of Tris-EDTA buffer containing SDS (1 mM Tris HCl, pH 8, 0.1 mM EDTA, 2% sodium dodecyl sulfate w/v), 140 µL of 5 M NaCl, and 70 µL of 10% CTAB at 60 °C. The mixture was vortexed for 2-3 secs followed by incubation for 60 min at 65 °C. To the incubated mixture, 600 µL of chloroform: isoamyl alcohol (24: 1 v/v) was added, mixed for 1 min, and centrifuged at 5000 rpm for 5 min at room temperature. The supernatant was separated into a new Eppendorf tube, and chloroform: isoamyl extraction was repeated. Then, the supernatant was transferred into a new Eppendorf tube while measuring the volume, and 0.2 volumes of 5 M NaCl and 2.5 volumes of 100% ethanol were added. The tube was gently mixed, kept at room temperature for 5 min, and centrifuged for 5 min at 13,000 rpm. After discarding the supernatant, the pellet was washed with 80% ice-cold ethanol. The pellet was dried for 30 min at room temperature and re-suspended in 50 µL of Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). The isolated DNA was visualized on a 1% agarose gel, and the rest of the sample was stored at -20 °C.

PCR amplification and sequencing of the ITS region

The *ITS*-1/5.8S rDNA/*ITS*-2 region of the genome was amplified using the *ITS1*/ forward primer: 5'-TCCGTAGGTGAACCTGCGG-3' and the *ITS4*/ reverse primer: 5'-TCCTCCGCTTATTGATATGC-3' (Fujita *et al.*, 2001). Ten microliters of the PCR reaction mixture were comprised of 5 µL of GoTaq® Green Master Mix [2X Green GoTaq® reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM MgCl$_2$], 3 µL of nuclease-free water, 0.5 µL of forward primer, 0.5 µL of reverse primer, and 1 µL of template DNA. An initial denaturation step at 94 °C for 30 seconds was followed by 45 cycles of double-stranded DNA denaturation for 30 seconds at 94 °C, annealing for 40 seconds at 55 °C, and 1 minute extension at 72 °C. Then, a final extension for 5 min at 72 °C was carried out. The amplified DNA sample was visualized in a 2% agarose gel with Ethidium Bromide as the stain. A 100 bp ladder was used to quantify the DNA band on a UV transilluminator. The PCR product was sequenced and compared with other sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Then, a phylogenetic tree was constructed using the sequences retrieved from GenBank database along with the sequence that showed 98% query coverage with the unknown sequence. The maximum likelihood statistical method with 1000 bootstrap replications and the Tamura-Nei model were used to infer the phylogenetic tree using the MEGA 7.0 tool.

Qualitative and quantitative screening of lipolytic activity

The lipolytic activity of the fungus was detected using the Tween 20 plate method. The medium was composed of: 1% peptone, 0.5% NaCl, 0.01% CaCl$_2$, 2% agar, and 1% Tween 20 as the lipid substrate. The pH of the medium was finally adjusted to 5.8 ± 0.2. The lipolytic activity of the isolated fungus was further confirmed by growing the fungal mycelia on culture plates with phenol red medium. The medium contained 0.01% phenol red as the indicator, 1% olive oil as the lipid substrate, 0.1% CaCl$_2$, and 2% agar. The pH was kept constant at 7.8 ± 0.2. Lipase activity was quantitatively determined using the para-nitrophenyl palmitate (pNPP) assay. Crude lipase activity was determined spectrophotometrically using pNPP as the substrate at 410 nm (Gupta *et al.*, 2002). In order to extract the crude lipase for the pNPP assay, the fungus was cultured for 3 days in a basal mineral medium, and then the medium was filtered through filter paper (Whatman No. 1) (Ülker *et al.*, 2011).

Optimization of lipase production medium (basal mineral medium)

The effect of the incubation period, carbon and nitrogen sources, and pH on the production of lipase by the fungus was studied. The best incubation period for the highest lipase secretion was determined by quantifying the lipase activity of lipolytic fungus for 5 consecutive days. The optimization of the carbon source was done using the following sources (1%): olive oil, coconut oil, vegetable oil, cellulose, margarine, and gingelly oil. The autoclaved media with different carbon sources were inoculated with the fungus. A separate set of flasks with the above carbon

sources in the liquid medium but without the fungus was maintained to detect changes in absorbance readings other than lipase activity. All media were incubated for 3 days at 32 °C in a shaking incubator at 120 rpm. Finally, the pNPP assay was used to measure lipase activity.

In order to study the effect of nitrogen sources on lipase secretion from the fungus, basal mineral medium was supplemented with different nitrogen sources (0.5% w/v): ammonium sulfate, peptone, ammonium nitrate, sodium nitrate, and urea. The rest of the procedure was conducted similarly to the carbon source optimization.

The effect of pH on the lipase secretion was evaluated by adjusting the pH of the basal mineral medium across a range of 3.0 to 10.0. The autoclaved medium, supplemented with optimized carbon and nitrogen sources, was adjusted to a different pH using concentrated HCl and NaOH before inoculating the fungus. For each pH value, a flask with uninoculated medium was maintained as a control. All media were incubated for 3 days at 32 °C in a shaking incubator at 120 rpm. The lipase activity was quantified using the pNPP assay.

Crude enzyme characterization

Enzyme characterization is crucial for identifying conditions that maximize enzyme activity. This can also reveal important features that can make the enzyme a better candidate for industrial applications. Therefore, the activity of *Trichoderma longibrachiatum* lipase was studied for pH, temperature, and cation requirements by changing the standard conditions of the pNPP assay.

Effect of pH and temperature on lipase activity

Using appropriate buffer solutions, the optimum pH of lipase activity was determined from 3.0 to 11.0. The lipase activity was measured at 40 °C by using 50 mM citrate phosphate buffer (pH 3.0-7.0), 50 mM Tris HCl (pH 8.0-9.0), and 50 mM sodium glycine (pH 10.0-11.0) in the pNPP assay. At each pH value, the reaction was carried out for 15 min. However, after the 15-min reaction, the pH of all the samples was adjusted to 8.0 using a constant volume of 1 M Tris HCl (pH 8.0) before measuring their absorbance at 410 nm.

The effect of temperature on lipase activity was investigated across the range of 20-70 °C by changing the temperature of the 15-min reaction time in the pNPP assay. The optimized pH recorded from the previous test was maintained during the 15-min reaction time. However, the pH of all the samples was adjusted to 8.0 using 1 M Tris HCl (pH 8.0) before recording the absorbance of samples.

Effect of cations on unchelated lipase activity

The effect of cations on the crude enzyme activity was examined by incubating 0.1 mL of the extracted crude enzyme with 0.1 mL of the following metal chlorides: $MgCl_2$, NaCl, KCl, $MnCl_2$, and $CaCl_2$ at 50 mM concentrations. The incubation was carried out for 60 min at room temperature. The remaining lipase activity was measured using a pNPP assay that was conducted under optimized assay conditions.

RESULTS AND DISCUSSION

Initial culturing of the fungus and its macroscopic and microscopic identification

The fungus was isolated from food waste-contaminated soil at the University of Peradeniya, Sri Lanka. The pure culture was flat, with a circular form and a filamentous margin. The colony growth was fast, and by the third day, white mycelium and green conidia formed a circular pattern, suggesting the genus of the lipolytic fungus as *Trichoderma* (Figure 1).
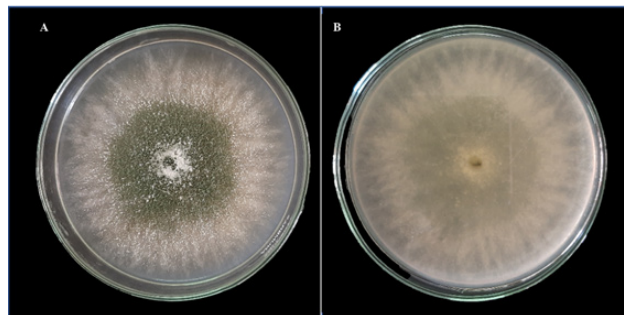

Figure 1: Morphology of the pure fungal colony on a PDA plate cultured by point inoculation. A: Top view of the culture plate. B: Bottom view of the culture plate.

Microscopic observations revealed that the mycelial branching of the fungus was bidirectional, the spores were spherical, and the green color of the colonies was caused primarily by the spores (Figure 2). Therefore, the microscopic features also agree with the characteristics of *Trichoderma* species.
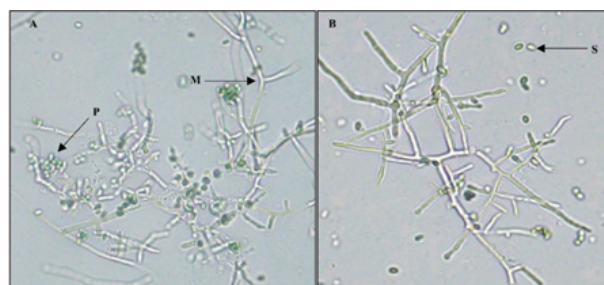

Figure 2: Microscopic view of *Trichoderma* sp.(×10 ×10). A: Represents mycelia (M) and clusters of spores (P). B: Indicates the spherical shape of the spores (S).

Molecular identification of the fungus

Species identification reveals important biochemical properties of the organism and helps screen new natural products. When considering fungi, classification based on morphology is problematic due to misleading morphological characteristics and dual nomenclature. Therefore, species identification using molecular techniques is crucial (Raja *et al.*, 2017). In this research, sequencing facilitates species-level identification that could be vital in reporting and introducing a specific fungus with lipolytic activity to industries. For the isolated genomic DNA, a clear band was observed in 1% agarose when the fungal sample was obtained from the growing edges of a 3-day old culture and after prolonged grinding and dissolving the final pellet in TE buffer. Molecular identification of fungi usually employs *ITS1* and *ITS4* primers that amplify the ITS-

1/5.8S rDNA/ITS-2 region (Fujita *et al.*, 2001). *ITS* regions evolve faster and exhibit high variation, allowing species-level identification (Raja *et al.*, 2017). For *Trichoderma longibrachiatum,* the amplified DNA fragment using the above primers resulted in a band of around 600 bp in 2% agarose. The sequence of the PCR product showed 100% identity with 98% query coverage and a 0.0 E value for *T. longibrachiatum* when a database search was conducted using the BLAST program. The inferred phylogenetic tree also indicated the clustering of the unknown sequence with the *T. longibrachiatum* sequence obtained from the GenBank database (Figure 3). Results from both molecular and morphological identification agreed that the lipolytic fungus is *T. longibrachiatum*.
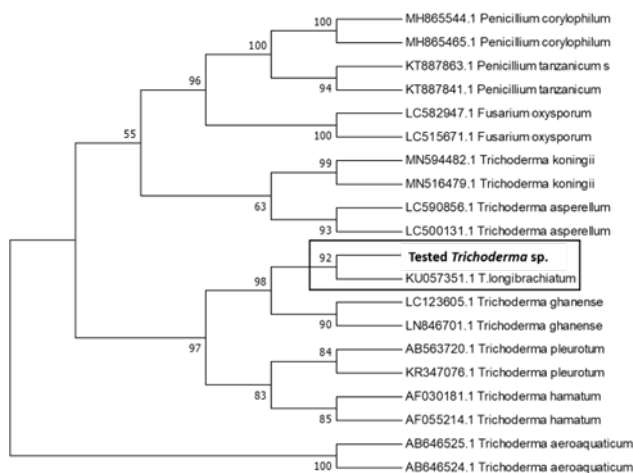

Figure 3: Shows the phylogenetic relationship of the unknown sequence to the selected sequences from the GenBank database. For the maximum likelihood gene tree based on the Tamura-Nei model, 1000 bootstrap replications were used. The black colored box indicates the clustering of tested species with *Trichoderma longibrachiatum*.

Qualitative and quantitative determination of lipolytic activity

The isolated *Trichoderma* sp. was screened for extracellular lipase using the Tween 20 and phenol red plate assays. In the Tween 20 plate assay, the white precipitate around the inoculum indicated the lipolytic activity of the fungus (Figure 4A). Fatty acids released by the hydrolysis of Tween 20 and calcium ions supplied in the medium form the insoluble fatty acid salt, which appears as a white precipitate (Lanka and Latha, 2015). Esters with medium-chain lengths are attacked rapidly by lipases with high affinity, making Tween 20 (C_{12}) a better substrate for lipase (Sakai *et al.*, 2002). However, the presence of lipase cannot be determined with the observations from the Tween 20 plate method alone since esterases hydrolyze Tween 20 and can give rise to similar observations (Tomioka, 1983). Therefore, an assay was conducted using phenol red as an indicator to detect the pH drop due to the hydrolysis of olive oil into fatty acids. This is a simple and rapid method for indicating lipase activity by a color change from red to yellow around the inoculum (Figure 4B). Also, this test can differentiate lipases from esterases since esterases give positive results only with tributyrin in the medium while lipases give positive results for both oil and tributyrin
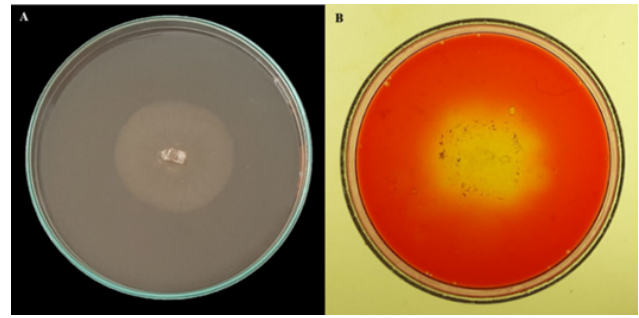
(Lanka and Latha, 2015).

Figure 4: Primary screening of lipolytic fungus, *Trichoderma* sp. by plate assays. A: Tween 20 plate with precipitation around the inoculum. B: Phenol red plate indicating yellow coloration around the inoculum.

The pNPP assay determines lipase activity quantitatively by employing pNPP as the substrate for lipase. Following the lipase reaction, the amount of para-nitrophenol (pNP) was estimated spectrophotometrically at 410 nm, and the resulting absorbance will be directly proportional to the lipolytic activity. Besides the fact that the method is simple and rapid, forming a turbid solution due to the immiscibility of pNPP or released fatty acids by lipase activity has to be overcome. For that, Triton X-100 is added to solubilize the fatty acids released by the enzymatic hydrolysis of pNPP (Gupta *et al.*, 2002).

Optimization of lipase production medium

The incubation period influences the microbial enzyme production notably, and it can extend from days to a week (Sharma *et al.*, 2017). For *Trichoderma longibrachiatum*, lipase production in the basal mineral medium increased drastically to reach a maximum at day 3, after which the lipolytic activity gradually declined (Figure 5).

The carbon and nitrogen sources were tested based on their cost, availability, composition, and results from previous studies. The highest lipase secretion was observed with olive oil as the carbon source. Further, olive oil contains nearly 80% oleic acid, a stabilizer for some extracellular lipases (de Almeida *et al.*, 2013). The lowest lipase secretion was observed in the absence of a carbon source, indicating the need for a carbon source for lipolytic fungal growth and subsequent lipase secretion (Figure 6A). Among different nitrogen sources, the highest lipase production was observed with ammonium sulfate. The absorbance reading with peptone was lower than the reading recorded without any nitrogen source in the medium, indicating an inhibitory effect of peptone on lipase production (Figure 6B). The current study did not use yeast extract as a nitrogen source since it is a complex hydrolysate of yeasts providing carbon, sulfur, growth factors, and other trace nutrients along with nitrogenous compounds.

The pH of the medium influences component transport through the cell membrane as well as enzymatic processes (Sharma *et al.*, 2017). For *Trichoderma longibrachiatum*, a gradual increase in lipolytic activity was observed when pH changed from 3.0 to 7.0. However, beyond pH 7.0, lipase secretion from the fungus declined drastically (Figure 7).
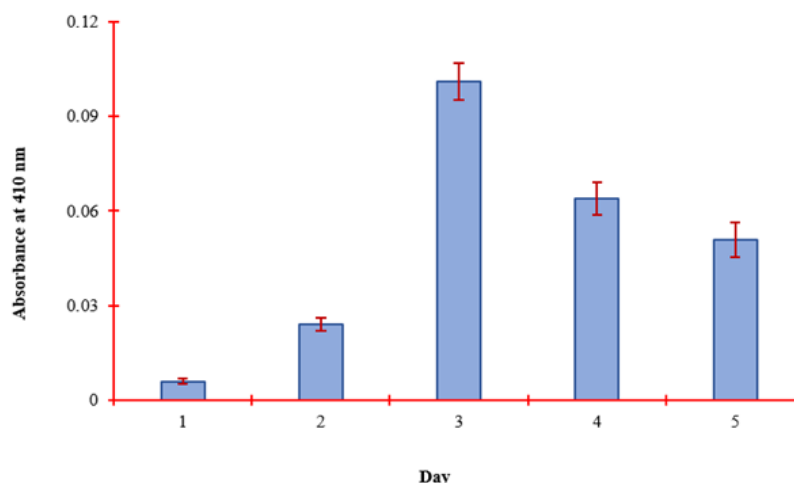
Figure 5: Quantitative determination of the lipase activity of the fungus, *Trichoderma* sp.. The histogram depicts the absorbance readings from the pNPP assay (y-axis) for 5 consecutive days (x-axis).
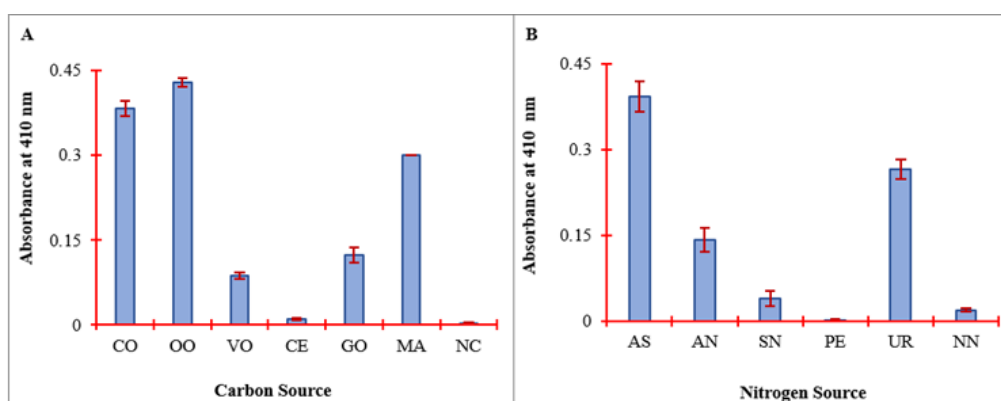

Figure 6: Optimization of the fungal growth medium for the Carbon and Nitrogen sources. A: Effect of different carbon sources on lipase secretion. CO: Coconut oil, OO: Olive oil, VO: Vegetable oil, CE: Cellulose, GO: Gingerly oil, MA: Margarine, and NC: No carbon source. B: Effect of different nitrogen sources on lipase secretion. AS: Ammonium sulfate, AN: Ammonium nitrate, SN: Sodium nitrate, PE: Peptone, UR: Urea, and NN: No nitrogen source.
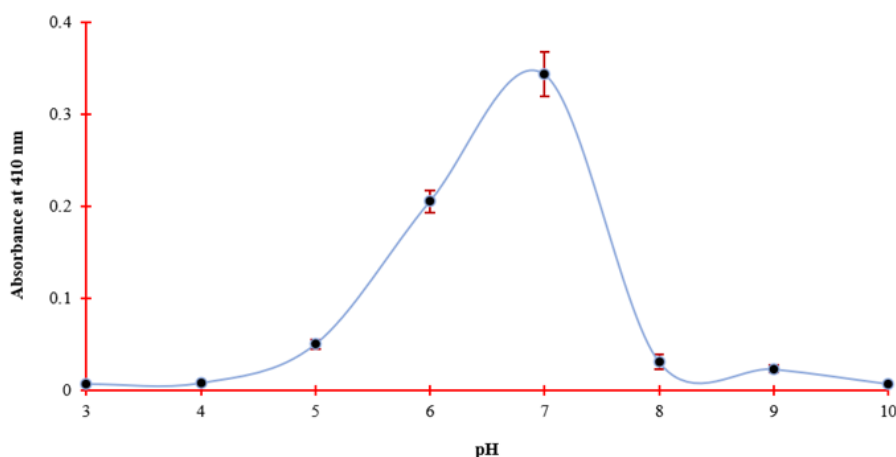

Figure 7: Effect of pH on lipase secretion from the lipolytic fungus, *Trichoderma* sp.. The line represents the relationship between absorbance measurements (y axis) and pH of the basal mineral medium (x axis).

Characterization of the enzyme

Changing pH affects the charges on amino acids, thus affecting the shape of the enzyme's active site. For the selected lipase, the activity increased with the increasing pH and reached a maximum at pH 6.0, followed by a drastic reduction in activity from pH 6.0 to 7.0, which then remained low until pH 9.0. Also, the extracted lipase was active under acidic conditions (Figure 8A). Previous studies have determined the lipase activities at each pH by obtaining absorbance at 348 nm without adjusting the pH to 8.0 (standard pH of the pNPP assay). 348 nm is the pH-independent isosbestic wavelength of pNP (Prive *et al*., 2013). However, in the current research, the final pH of the reaction mixture was adjusted to pH 8.0, and then the absorbance was recorded at 410 nm since the 410 nm

wavelength gives high sensitivity. When considering the temperature, lipase activity increased with the temperature to reach a peak at 40 °C, followed by a gradual drop when the reaction temperature was increased beyond 40 °C (Figure 8B). High temperatures lead to protein denaturation, disrupting the shape of the active site and reducing enzyme activity. However, for the tested lipase. a considerable amount of enzymatic activity was observed at high temperatures as well. This feature makes the enzyme an attractive candidate for industrial applications where processes involve high temperatures.

Lipases are known as metalloenzymes; thus, they usually require metal ions in their active site to perform catalytic activity (Lestari *et al*., 2016). For *Trichoderma longibrachiatum*-secreted lipase, the highest enzyme activity was observed in the presence of Ca^{2+}, and this can be due to a stabilizing effect on enzyme conformation. Meanwhile, Mg^{2+} and Mn^{2+} moderately activated the enzyme (Figure 9). However, Na^+ and K^+ exhibited inhibitory effects on the enzyme. These results indicated the possible activation of enzyme activity by preincubating with Ca^{2+}.
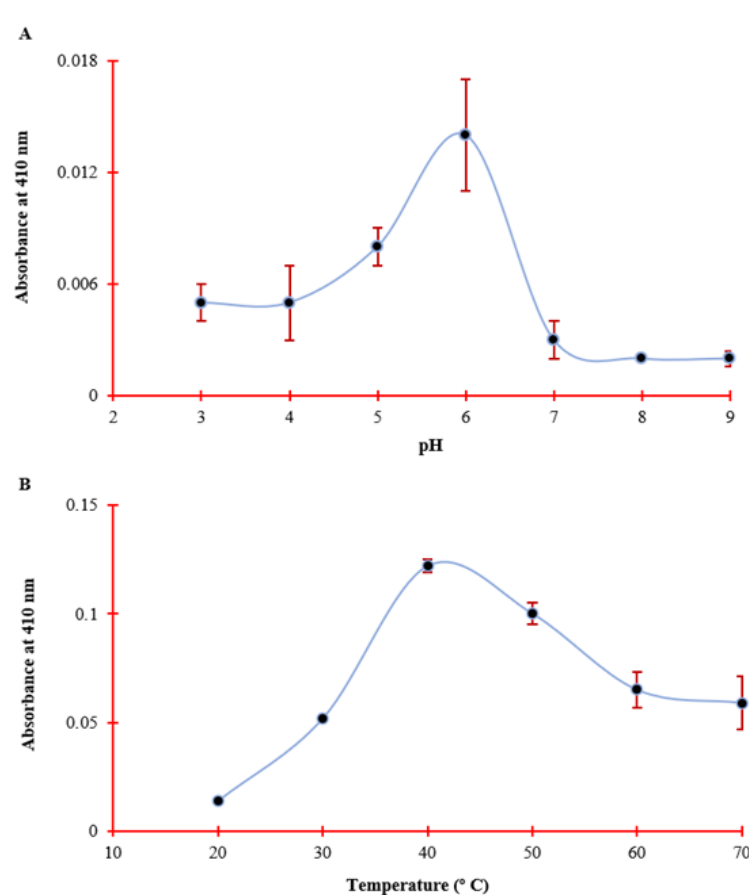

Figure 8: Impact of pH and temperature on lipase activity of the fungus, *Trichoderma* sp.. A: Lipase activity across the pH range of 3.0 to 9.0 (x-axis) with absorbance measurements at 410 nm (y-axis). B: Dependence of lipase activity on temperature in the range of 20 to 70 °C (x-axis), with absorbance measurements at 410 nm (y-axis).
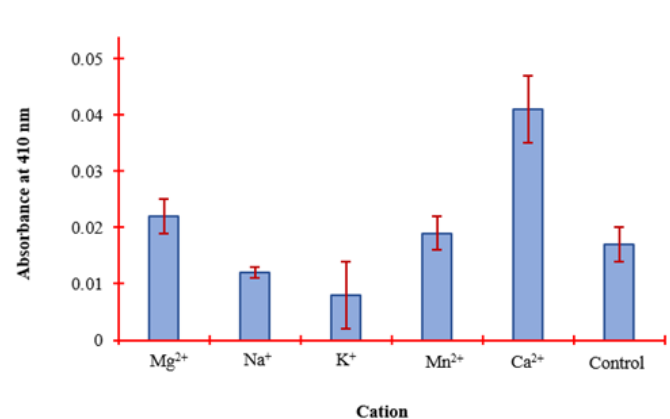

Figure 9: Effect of cations on lipase activity of the fungus, *Trichoderma* sp.. A: Variation of unchelated lipase activity with different cations (x-axis) analyzed by absorbance measurements at 410 nm (y-axis).

CONCLUSIONS

The current research reveals that *Trichoderma longibrachiatum* is industrially favorable due to its high growth rate and requirement of less costly but widely available culture components and conditions. This study also revealed that the lipase secretion from *T. longibrachiatum* could be increased by optimizing the carbon and nitrogen sources and pH of the growth medium. As observed, olive oil and ammonium sulfate, as the carbon and nitrogen sources, respectively, at pH 7.0, yield a high lipase secretion. Moreover, the characterization experiments revealed that the lipase activity could be enhanced under optimized conditions. Lipase activity showed a significant dependence on pH and temperature. The maximum enzyme activity was reported at a pH of 6.0 and a temperature of 40 °C. Because the lipase activity is significant in acidic conditions, this lipase is a good candidate for the oleochemical industry. Retention of activity at high temperatures makes the enzyme a potential candidate for industrial applications. Moreover, the enzyme activity could be enhanced using Ca^{2+}. The study identifies optimized conditions for several important parameters affecting enzyme activity and will be a foundation for future research exploring other factors that affect the lipase activity, such as activators, inhibitors, and the stability of the enzyme in the presence of industrially used chemicals. Furthermore, improving the strain by modifying the fungus' genetic makeup can allow higher enzyme secretion and activity.

DECLARATION OF CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

BCC Research (2018 Dec). *Global lipase market*. DOI: https://www.bccresearch.com/partners/verified-market-research/global-lipase-market.html.

Boratyński, F., Szczepańska, E., Grudniewska, A., Gniłka, R. and Olejniczak, T. (2018). Improving of hydrolases biosythesis by solid-state fermentation of *Penicillium camemberti* on rapeseed cake. *Scientific reports* **8**(1): 1-9. DOI: 10.1038/s41598-018-28412-y.

Burkert, J.F.D.M., Maldonado, R.R., Filho, F.M. and Rodrigues, M.I. (2005). Comparison of lipase production by *Geotrichum candidum* in stirring and airlift fermenters. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology* **80**(1): 61-67. DOI: 10.1002/jctb.1157.

Daiha, K., Angeli, R., de Oliveira, S. D. and Almeida, R. V. (2015). Are lipases still important biocatalysts? A study of scientific publications and patents for technological forecasting. *PloS one* **10**(6): e0131624. DOI: https://doi.org/10.1371/journal.

de Almeida, A. F., Taulk-Tornisielo, S. M. and Carmona, E. C. (2013). Influence of carbon and nitrogen sources on lipase production by a newly isolated Candida viswanathii strain. *Annals of Microbiology* **63**(4), 1225-1234. DOI: https://doi.org/10.1007/s13213-012-0580-y.

Fujita, S. I., Senda, Y., Nakaguchi, S., and Hashimoto, T. (2001). Multiplex PCR using internal transcribed spacer 1 and 2 regions for rapid detection and identification of yeast strains. *Journal of clinical microbiology*, **39**(10), 3617-3622. DOI: https://doi.org/10.1128/JCM.39.10.3617-3622.2001.

Griffith, G.W., Easton, G.L., Detheridge, A., Roderick, K., Edwards, A., Worgan, H.J., Nicholson, J. and Perkins, W.T. (2007). Copper deficiency in potato dextrose agar causes reduced pigmentation in cultures of various fungi. *FEMS microbiology letters* **276**(2): 165-171. DOI: https://doi.org/10.1111/j.1574-6968.2007.00923.x.

Gulati, R., Isar, J., Kumar, V., Prasad, A.K., Parmar, V.S. and Saxena, R.K. (2005). Production of a novel alkaline lipase by *Fusarium globulosum* using neem oil, and its applications. *Pure and Applied Chemistry* **77**(1): 251-262. DOI: 10.1351/pac200577010251.

Gupta, N., Rathi, P. and Gupta, R. (2002). Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Analytical Biochemistry* **311**(1): 98-99. DOI: https://doi.org/10.1016/s0003-2697(02)00379-2.

Gupta, R., Kumari, A., Syal, P. and Singh, Y. (2015). Molecular and functional diversity of yeast and fungal lipases: their role in biotechnology and cellular physiology. *Progress in Lipid Research* **57**:40-54. DOI: 10.1016/j.plipres.2014.12.001.

Harman, G.E. (2006). Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**(2): 190-194. DOI: https://doi.org/10.1094/PHYTO-96-0190.

Hasan, F., Shah, A.A. and Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial technology* **39**(2): 235-251. DOI: https://doi.org/10.1016/j.enzmictec.2005.10.016.

Helal, S. E., Abdelhady, H. M., Abou-Taleb, K. A., Hassan, M. G. and Amer, M. M. (2021). Lipase from *Rhizopus oryzae* R1: in-depth characterization, immobilization, and evaluation in biodiesel production. *Journal of Genetic Engineering and Biotechnology*, **19**(1), 1-13. DOI: https://doi.org/10.1186/s43141-020-00094-y.

Jaeger, K.E. and Eggert, T. (2002). Lipases for biotechnology. *Current Opinion in Biotechnology* **13**(4):390-397. DOI: https://doi.org/10.1016/S0958-1669(02)00341-5.

Lanka, S. and Latha, J.N.L. (2015). A short review on various screening methods to isolate potential lipase producers: lipases-the present and future enzymes of biotech industry. *International Journal of Biological Chemistry* **9**(4): 207-219. DOI: https://doi.org/10.3923/IJBC.2015.207.219.

Leck A. (1999). Preparation of lactophenol cotton blue slide mounts. *Community Eye Health*. **12**(30):24.

Lestari, P., Raharjo, T.J., Matsjeh, S. and Haryadi, W. (2016). Partial purification and biochemical characterization of extracellular lipase from *Azospirillum* sp. JG3 bacteria. In *AIP Conference Proceedings* **1755**(1): 080003. DOI: 10.1063/1.4958511. DOI: https://doi.org/10.1063/1.4958511.

Liu, G., Hu, S., Li, L. and Hou, Y. (2015). Purification and characterization of a lipase with high thermostability and polar organic solvent-tolerance from Aspergillus

niger AN0512. *Lipids*, *50*(11), 1155-1163. DOI: https://doi.org/10.1007/s11745-015-4052-6.

Mehta, A., Bodh, U. and Gupta, R. (2017). Fungal lipases: a review. *Journal of Biotech Research* **8**(1)**:** 58-77.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J. and Sussman, J.L. (1992). The α/β hydrolase fold. *Protein Engineering, Design and Selection* **5**(3): 197-211. DOI: https://doi.org/10.1093/protein/5.3.197.

Prive, F., Kaderbhai, N.N., Girdwood, S., Worgan, H.J., Pinloche, E., Scollan, N.D., Huws, S.A. and Newbold, C.J. (2013). Identification and characterization of three novel lipases belonging to families II and V from *Anaerovibrio lipolyticus* 5ST. *PLoS One* **8**(8): e69076. DOI: 10.1371/journal.pone.0069076.

Raja, H. A., Miller, A. N., Pearce, C. J. and Oberlies, N. H. (2017). Fungal identification using molecular tools: a primer for the natural products research community. *Journal of natural products* **80**(3), 756-770. DOI: https://doi.org/10.1021/acs.jnatprod.6b01085.

Sakai, Y., Hayatsu, M. and Hayano, K. (2002). Use of Tween 20 as a substrate for assay of lipase activity in soils. *Soil Science and Plant Nutrition* **48**(5): 729-734. DOI: https://doi.org/10.1080/00380768.2002.10409263.

Schmid, R.D. and Verger, R. (1998). Lipases: Interfacial enzymes with attractive applications. *Angewandte Chemie International Edition* **37**(12): 1608-1633. DOI: https://doi.org/10.1002/(SICI)1521-3773(19980703)37:12%3C1608::AID-ANIE1608%3E3.0.CO;2-V.

Shahid, M., Singh, A., Srivastava, M., Mishra, R.P. and Biswas, S.K. (2011). Effect of temperature, pH and media for growth and sporulation of *Trichoderma longibrachiatum* and shelf -life study in carrier-based formulations. *Annals of Plant Protection Science* **19**(1): 147-147.

Sharma, K. M., Kumar, R., Panwar, S. and Kumar, A. (2017). Microbial alkaline proteases: Optimization of production parameters and their properties. *Journal of Genetic Engineering and Biotechnology* **15**(1): 115-126. DOI: https://doi.org/10.1016/j.jgeb.2017.02.001.

Sharma, R., Chisti, Y. and Banerjee, U.C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology advances* **19**(8): 627-662. DOI: https://doi.org/10.1016/S0734-9750(01)00086-6.

Tomioka, H.A.R.U.A.K.I. (1983). Purification and characterization of the tween-hydrolyzing esterase of Mycobacterium smegmatis. *Journal of bacteriology* **155**(3): 1249-1259. DOI: https://doi.org/10.1128%2Fjb.155.3.1249-1259.1983.

Ülker, S., Özel, A., Çolak, A. and Karaoğlu, Ş.A. (2011). Isolation, production, and characterization of an extracellular lipase from *Trichoderma harzianum* isolated from soil. *Turkish Journal of Biology* **35**(5): 543-550. DOI: http://dx.doi.org/10.3906/biy-1004-107.

Wang, Y., Ma, R., Li, S., Gong, M., Yao, B., Bai, Y. and Gu, J. (2018). An alkaline and surfactant-tolerant lipase from *Trichoderma lentiforme* ACCC30425 with high application potential in the detergent industry. *AMB Express* **8**(1): 1-11. DOI: https://doi.org/10.1186%2Fs13568-018-0618-z.